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Availability of specific sugars for glycoconjugate biosynthesis: A need for further investigations in man

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Summary — We review the metabolism of specific sugars used for protein glycosylation, focusing on the fate of exogenously provided sugars. Theoretically, all glycoprotein sugars can derive from glucose, but previous studies show that other exogenous sugars can be incorporated into glycoproteins. From data obtained in congenital galactosemia, exogenous galactose may be important for correct glycosylation. Contrary to galactose, the metabolism of other sugars seems to depend on insulin regulation: stimulation of their endogenous production in diabetic subjects might participate in some diabetic complications, precluding the need for an exogenous supply. The metabolic fate of these sugars is different according to the administration route and exogenous supply may be important either in enteral nutrition or in some clinical situations as has been suggested for sialic acid in the newborn. Data in man are too sparse to reach firm conclusions, implying a need for further investigations. Our preliminary results in animals and man demonstrate that stable isotope methodology allows one to trace glycoprotein sugar metabolism in nutritionally relevant conditions with accuracy and sensitivity, using doses of specific sugars well below toxic levels. (© Société française de biochimie et biologie moléculaire / Elsevier, Paris).

dietary sugars / glycoprotein biosynthesis / stable isotopes

Introduction

Glycoproteins are ubiquitous in mammals; their glycan moieties exhibit diverse important functions, from non-specific roles in protein structure and stability to very specific ones in signal recognition. The carbohydrate chains are synthesized by a non-template mechanism, for which expression of specific glycosyltransferases in subcellular compartments clearly plays a major regulatory role. However, despite a high level of genetically determined control, glycans display a range of structural microheterogeneity in a given individual. As a source of this microheterogeneity, the availability of specific sugar-nucleotides, obligatory substrates for glycosyltransferases, could represent an alternative control mechanism which has received little attention and depends on the metabolism of two moieties, sugar and nucleotide. Nutritional factors (quantity or quality of various nutrients) can induce modifications in glycosylation processes [1]. A part of the reported effects of nutrients relies on nucleotide availability: for example, protein intake regulates nucleotide availability [2] and nucleotide availability could influence glycosylation [3]. In clinical practice, antimetabolites interfering with nucleotide metabolism could exert a part of their cytotoxic effects by altering glycosylation [4]. Another part of the effects of nutrition could depend on specific sugar availability, which will be the focus of this review. Theoretically, all glycoprotein sugars can derive from glucose. However, it is known that specific sugars could be more or less efficiently incorporated into glycoproteins, but it is not known whether exogenous specific sugars could be preferential substrates for glycosylation or have a metabolic usefulness or exert regulatory properties in glycosylation processes. In the last part of this paper, we present preliminary results in man, demonstrating that stable isotope methodology could be applied to the study of glycoprotein sugar metabolism in nutritionally relevant conditions.

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Nutritionally relevant data on sugar metabolism in relation to glycoprotein glycosylation

The general picture of the metabolism of specific sugars for glycosylation is shown in figure 1. Close contact points with major metabolic pathways of important nutrients are obvious, but the physiological significance of such interactions is still poorly understood.

Galactose

Galactose has been the most studied non-glucose sugar since, *via* lactose from dairy products, it is abundant in the diet and provides substrate for oxidation or glycogen syn-

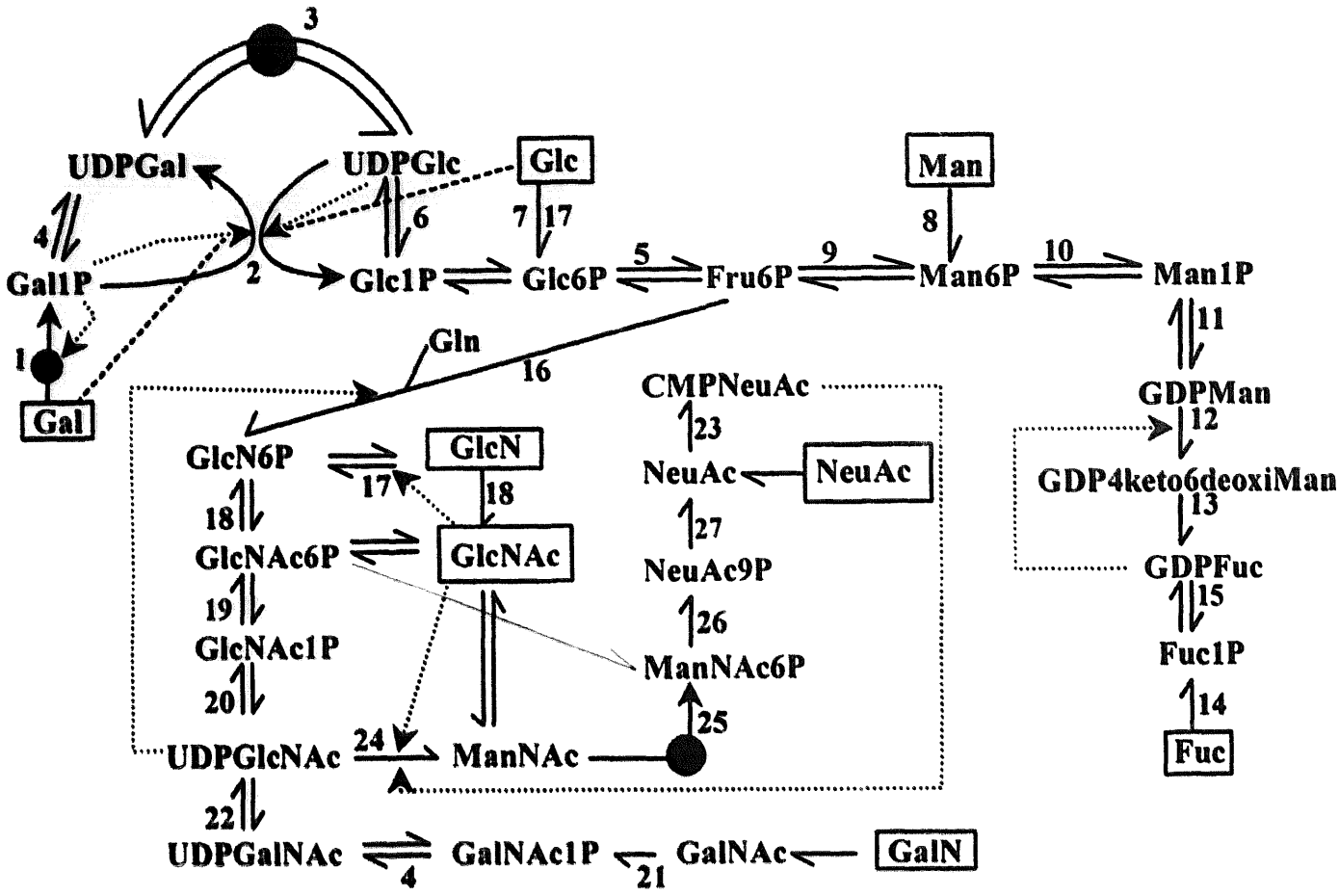

Fig 1. Simplified general scheme of the metabolism of specific sugars. Enzymes are numbered consecutively according to their order of appearance in the text. Sugars in boxes can be supplied by the diet. Thin line: minor route. *In vitro* regulation of enzymatic activities by substrates or products: dotted lines, inhibition; dashed lines, activation. Full circles on arrows indicate suggested *in vivo* rate-limiting steps. 1, Galactokinase EC 2.7.1.6; 2, galactose-1-phosphate uridyltransferase EC 2.7.7.12; 3, UDP-galactose 4-epimerase EC 5.1.3.2; 4, UDP-galactose pyrophosphorylase EC 2.7.7.10; 5, phosphoglucose isomerase EC 5.3.1.9; 6, UDP-glucose pyrophosphorylase EC 2.7.7.9; 7, hexokinase EC 2.7.1.1; 8, mannokinase EC 2.7.1.7; 9, phosphomannose isomerase EC 5.3.1.8; 10, phosphomannomutase EC 5.4.2.8; 11, GDP-mannose pyrophosphorylase EC 2.7.7.13; 12, GDP-mannose 4,6 dehydratase EC 4.2.1.47; 13, GDP-4-keto,6-deoximannose epimerase-reductase EC 1.1.1.187; 14, fucokinase EC 2.7.1.52; 15, GDP-fucose pyrophosphorylase EC 2.7.7.30; 16, glutamine: fructose-6-phosphate amidotransferase EC 2.6.1.16; 17, glucokinase EC 2.7.1.2; 18, glucosamine-6-phosphate acetyltransferase EC 2.3.1.4; 19, N-acetylglucosamine-6-phosphate mutase EC 2.7.5.2; 20, UDP-N-acetylglucosamine pyrophosphorylase EC 2.7.7.23; 21, N-acetylgalactosamine kinase, EC not yet attributed; 22, UDP-N-acetylgalactosamine 4-epimerase EC 5.1.3.7; 23, CMP-N-acetylneuraminic acid synthase EC 2.7.7.43; 24, UDP-N-acetylglucosamine 2-epimerase EC 5.1.3.14; 25, N-acetylmannosamine kinase EC 2.7.1.60; 26, N-acetylneuraminic acid 9-P synthase EC 4.1.3.20; 27, N-acetylneuraminic acid 9-phosphatase EC 3.1.3.29.

thesis, while the existence of galactosemia by deficiency in galactose-1-phosphate uridyltransferase activity has led to research which is relevant to glycoprotein synthesis.

Galactose is well absorbed from the intestine by glucose facilitative transporters SGLT1 and GLUT1 [5]. Despite common transporters, there is evidence in man that competition for absorption between galactose and glucose is not the single determinant of the reduced galactosemia observed after concomitant ingestion of galactose and glucose as compared to ingestion of galactose alone [6]. Galactose absorption is modulated by many factors such as antibiotics [7] which are not relevant to current nutrition, so that galactose absorption is used as a marker for the effect of a drug on intestinal absorption. The presence of glucose accelerates galactose removal from blood [6, 8, 9] which is performed essentially by the liver [8, 10, 11] although some other organs such as kidney [12] or intestine can also utilize this sugar. Non-metabolized galactose is excreted in the

urine with no evidence of a threshold [13]. The predominance of galactose metabolism in the liver has led to the study of galactose clearance as a marker for liver dysfunction: thus, galactose clearance is predictive of death in cirrhotic patients [14]. Galactose is converted to a large extent into glucose in the Leloir pathway which has been reviewed elsewhere [15–17] so that only data relevant to our objective will be briefly indicated.

Galactokinase (#1 in fig 1) proceeds *via* a sequential reaction, non-competitively inhibited by galactose-1-phosphate and competitively by Mg-ADP [18]. In HepG2 cells, the presence of galactose in the medium decreases the activity of the enzyme which could be the rate-limiting enzyme in the pathway [19].

The second enzyme in the Leloir pathway is galactose-1-phosphate uridyltransferase (#2), which produces UDP-galactose and glucose-1-phosphate from galactose-1-phosphate and UDP-glucose. The enzyme has been cloned [20] and is deficient in galactosemia. Its expression is developmentally regulated [21, 22]. It is activated by glucose [23] and by the presence of galactose, either in the culture medium for HepG2 cells [19], or in the diet [24] or in intravenous perfusion especially in the young where the activation is enhanced by feeding folic acid [25]. In HepG2 cells, the regulation by galactose appears to be located at a post-transcriptional level and correlates to cell growth [26]. Enzyme activity is sensitive to substrate inhibition by UDP-glucose or galactose-1-phosphate and is also inhibited by various uridine nucleotides [16]. This enzyme consists of a dimer [27], each catalytic site bearing a single binding site, so that it follows a ping-pong mechanism [17].

The third enzyme in the pathway is UDP-galactose epimerase (#3), which has two functions. In presence of an excess of exogenous galactose, it provides UDP-glucose for the function of the uridyltransferase; like the transferase, it is inhibited by UDP-glucose and uridine nucleotides [16]. Conversely, in the absence of exogenous galactose, this enzyme is responsible for endogenous formation of galactose from glucose, which is around 0.5–1.05 mg/kg h in man in the fasted state, at the same level in normal or galactosemic subjects [28]. This production could account for galactose toxicity in galactosemic subjects who cannot recycle endogenously formed galactose and provides an alternative explanation to the hypothesis that toxicity could arise from hidden sources of galactose in the diet [29].

Three other enzymes could be involved in the metabolism of galactose [16], but their physiologic importance is not known in normal man, though they could be important in galactosemic subjects [30]: UDP-galactose pyrophosphorylase (#4) is high in the neonatal period, then decreases to 1/100 of uridyltransferase activity [16]; phosphoglucose-isomerase (#5) is able to produce galactose-6-phosphate; UDP-glucose pyrophosphorylase (#6) could also react with galactose-1-phosphate and produce UDP-galactose.

Via these pathways, galactose enters three major metabolic routes: i) incorporation into glycogen: the synthesis of glycogen from galactose, though less efficient than from glucose [31], uses up to 30% of exogenous galactose [32], the epimerase step being likely the rate-limiting step for this synthesis. The drug acetaminophen can be used to trace the label of the precursor pool UDP-galactose used in glycogen or glycoprotein synthesis since acetaminophen is conjugated with glucuronic acid derived from glucose or galactose and the conjugate eliminated in the urine [9, 33]; ii) oxidation in the glycolytic pathway: the oxidation rate of galactose is limited as compared to glucose and reaches only 50% that of glucose during physical exercise [34]. In the fasting state in man, 27–47% of galactose in tracer amounts (7 mg/kg) are converted to carbon dioxide within 5 h [35]. This proportion of oxidation does not seem to be modified with a larger load [36]; and iii) since all the exogenous galactose is not found in these two pathways, there must be a third pathway, glycoconjugate biosynthesis using UDP-galactose as substrate of glycosyltransferases. A specific transporter moving UDP-galactose across Golgi membrane for this purpose has been cloned [37]. Perhaps due to the existence of oxidation and the incorporation into glycogen, labeled galactose has been very little used for the study of glycoprotein metabolism.

Interesting data have been obtained by the study of 'classic' galactosemic patients carrying uridyltransferase deficiency. UDP-galactose is decreased in red blood cells of galactosemic subjects [38, 39] so that the ratio UDP-glucose/UDP-galactose increased from the normal value of around 3 to 3.8–4.2; the concentration of galactose-1-phosphate increased in parallel. The decrease in UDP-galactose could explain the low incorporation of galactose into the glycoconjugates of fibroblasts and the decrease of galactolipids in the brain of affected subjects [40] despite a correct elimination of galactose from the diet. Indeed, in non-galactosemic subjects, galactose provided by the diet (at least 3 g/day) is necessary to achieve a normal ratio of nucleotide-sugars in red blood cells, whereas dietary glucose does not affect this ratio [41, 42]. Galactose provided by dietary oligosaccharides such as stachyose or raffinose can also be used as galactose sources since their elimination from the diet tends to decrease galactose-1-phosphate concentrations in red blood cells of galactosemic subjects [43]. Galactose containing oligosaccharides are absorbed in the colon of the pig, more efficiently in the young than in the adult [44]. It is noteworthy that the addition of uridine in the patient diet or in the medium of cell cultures has also positive effects on sugar-nucleotide pools though it inhibits *in vitro* the enzymes of the Leloir pathway.

Contrasting with mannose or glucosamine, serum galactose is not modified in diabetes mellitus [45], since insulin is not necessary for a normal response after a galactose load [6] and galactose perfusion does not stimulate insulin secretion in man [46].

Excessive galactose ingestion could result in cataract development, which is explained by action of aldose reductase producing galactitol, which displays osmotic properties [47] and glycation properties responsible for cataract [48]. It has also been proposed that it could be a dietary factor in ovarian cancer [49], though galactose consumption and metabolism do not seem to be associated to ovarian senescence [50]. It could have also a calculogenic potential, especially in the case of vitamine B6 deficiency, by increasing calcium oxalate excretion [51].

Perioperative galactose perfusion (1.9 g/kg body weight per day) has recently been shown to partially prevent metastasis from stomach or colon cancers in surgical patients [52]. Though the suggested mechanism relies on inhibition by galactose of lectin activities, a role on glycoconjugate synthesis and structural microheterogeneity can not be ruled out.

Mannose

Though data on mannose are less abundant than for galactose, it has been shown that most of the mannose for N-glycosylation of glycoproteins in cultured normal human fibroblasts under physiological conditions derives from mannose rather than from glucose [53].

Mannose is absorbed from the gut less efficiently than glucose (15–20% of the glucose rate) [54]. Specific transporters for mannose have been described in established cell lines from colon adenocarcinoma: Caco-2 and HT-29 cells possess a transporter inhibited by deoxymannojirimycin [55, 56] while Caco-2 cells possess a second transporter insensitive to this drug [55]. These transporters are clearly distinct from glucose transporters. As a result, *in vivo* in man, there is no competition of glucose or sucrose for mannose absorption [57]; at dosages higher than 0.2 g/kg body weight, the subjects experienced gastrointestinal symptoms [57, 58]. Serum mannose peaks at 90 min after oral ingestion and is slowly eliminated from the blood (half-time 4 h) by a specific transporter [59] insensitive to glucose, the K_m of which is close to the normal serum concentration of mannose (50 µM [60]).

Inside the cell, mannose is phosphorylated to mannose-6-phosphate by hexokinase (#7) or mannokinase (#8). Mannose-6-phosphate enters the glycolytic pathway after action of phosphomannose isomerase (#9) giving fructose-6-phosphate. This enzyme allows endogenous production of mannose from glucose as well as oxidation of exogenous mannose. Back and forth fluxes through the enzyme represent 4% of glucose flux in red blood cells and reach 47% in pancreatic islets [61], so that modelization of glucose metabolism must take into account this activity [62]. The enzyme has been cloned in man: it is expressed in all tissues, but at a high level in heart, brain and skeletic muscle [63]. It is strongly inhibited by fructose-1-phosphate, which can lead to alterations in protein glycosylation in hereditary fructose intolerance [64]. By means of this enzyme, mannose can be an excellent substrate to alleviate hypoglycemic symptoms [65] or for brain cell metabolism [66].

For utilization in glycoprotein biosynthesis, mannose-6-phosphate is converted to mannose-1-phosphate by phosphomannomutase (#10), which is encoded by two genes: PMM1 (located on chromosome 22 [67]) and PMM2 (located on chromosome 16 [68]). Mutations affecting this second gene [68] are responsible for the deficiency in phosphomannomutase activity observed in carbohydrate deficient glycoprotein syndrome type I [69]. Exogenous mannose can efficiently correct *in vitro* aberrant glycosylation in the cells obtained from affected patients [70]. Finally, mannose-1-phosphate is converted to GDP-mannose by GDP-mannose pyrophosphorylase (#11), which has been recently purified from pig liver [71].

In man [46], as in the perfused pancreas [72], mannose leads to secretion of preformed insulin, but does not induce insulin synthesis [72]. In diabetes mellitus, the ratio of mannose to glucose is increased in the serum and this increase could be involved in diabetic complications [73]. Increases in serum mannose concentrations have also been observed in nephropathies [74].

Fucose

Endogenous fucose is produced directly in the sugar-nucleotide form (GDP-fucose) from GDP-mannose, by a route delineated more than 25 years ago by Ginsburg [75], consisting of two enzymes, GDP-mannose 4,6 dehydratase (#12) [76] and an epimerase-reductase (#13) [77] working with NADPH. Cloning of a homodimeric NADPH-binding protein purified from red blood cells revealed that it was this last enzyme [78].

Recycled or exogenous fucose is converted to fucose-1-phosphate by fucokinase (#14) [79], then to GDP-fucose by a pyrophosphorylase (#15) [80]. Inhibitors of fucokinase lower fucose incorporation into glycoproteins [81]. Ingested or injected radioactive fucose is found in glycoproteins [80]. In man, a large proportion of injected fucose is oxidized (39% [83]) contrasting with a low oxidation rate after injection in the rat (1.6% [84]). In the rat, oral ingestion leads to a larger catabolism, which comes in part from intestine bacterial activity [82]. The addition to the tracer of a large amount of non-radioactive fucose strongly increases fucose urinary excretion [85]. Kinetic studies of tissue labeling after injection in the rat suggest the existence of fucose recycling for glycosylation [84]. Recycling has also been demonstrated in cell cultures [86]. Many cells possess a specific facilitative transporter for fucose [87]. Exogenous fucose can also be provided by fucose-containing glycoproteins present in the diet [82].

Free fucose is found in low concentrations in serum, which is increased in cancerous and diabetic patients [88]. High concentrations could induce perturbation in cellular uptake of myoinositol (resulting in altered phosphoinositide metabolism and signaling) and reversible disturbances in

nervous conduction [89]. Alterations of collagen synthesis by exogenous fucose in endothelial cells has also been described [90]. As for mannose, such alterations could be involved in diabetic complications, though the concentrations used in some of these studies (20% fucose in the diet or 30 mM fucose concentration in cell culture medium) raise doubt on the physiological relevance of the effects.

Glucosamine

Endogenous glucosamine is produced from glucose by amination of fructose-6-phosphate. There has been recent renewal of interest for this pathway since the discovery by Marshall of the implication of the glucosamine pathway in the mechanisms of insulinoresistance (see [91, 92] for review). Though it is known from the work of Kornfeld [93] that the final product of the pathway (UDP-GlcNAc) inhibits the first enzyme of the pathway, glutamine: fructose-6-phosphate amidotransferase (GFAT, #16), increased fluxes through this enzyme clearly mediate a part of glucose toxicity in diabetic subjects. *In vitro* and *in vivo* studies support this conclusion, such as those using GFAT transgenic mice [94], GFAT determinations in muscle of diabetic patients [95] or glucosamine infusion in the rat [96, 97].

Some of the mechanisms underlying this effect have been delineated in insulin-sensitive tissues, notably the alteration of Glut4 translocation [98]. *In vivo*, glucosamine inhibits glucokinase and decreases insulin secretion [99] whereas hepatic glucose production is not suppressed [100]. Moreover, there are alterations of UDP-sugar pools which could lead to alterations of glycoprotein synthesis [101]. In the same way, the addition of tunicamycin, a well known inhibitor of protein glycosylation, suppresses the ability of glucose to stimulate TGFα activity [102], while the transformation of glucose to glucosamine is needed for activation of TGFα transcription [103]. However, increased fluxes through the enzyme require hyperglycemia and there is no proof that glucosamine is the primary mechanism for insulinoresistance: glucosamine signaling appears to be a normal regulatory role, the amplification of which leads to pathological consequences in diabetes [92].

On the other hand, it has been suggested that exogenous glucosamine could be beneficial in some pathological conditions such as wound healing [104] or osteoarthritis [105]: some clinical reports indicated that oral glucosamine alleviates symptoms and pain in osteoarthritis. Thus, glucosamine is already sold as a dietary supplement in the US and also as a drug in the form of the prodrug glucosamine sulfate [106]. The suggested mechanism of such actions relies on intervention of glucosamine in proteoglycan synthesis. Conversely, deprivation of glutamine to chondrosarcoma cells in culture decreases the pool of UDP-hexosamines, which is worsened by the addition of diazonorleucin, an inhibitor of GFAT activity [107].

Though there is no report on the possible relation of oral glucosamine supply and insulino-resistance, one might argue that there are striking differences in the metabolic fate of glucosamine according to the administration route, parenteral or enteral, which has been reported many years ago in the rat or the rabbit [108–113] and recently confirmed with the prodrug glucosamine sulfate in man [106]. After oral ingestion, about half of the dose is oxidized and less is found in glycoproteins (except for the gastrointestinal tract) as compared to injected glucosamine [108], although in another study, only 4% of oral glucosamine was oxidized in neomycin-treated rats [113]. These studies have established that the absorption rate of glucosamine is around 1/10 that of glucose [113, 114] and that oxidation by bacterial microflora can occur, which cannot completely account for the differences between intravenous and oral routes [108, 113]. N-acetylglucosamine appears to be a less efficient precursor than glucosamine for glycosylation [115], in part because of a low cellular uptake [116]. As in the case of fucose, protein-bound glucosamine in the diet can be used by the organism for glycosylation [108, 117].

Contrasting with endogenous glucosamine which is produced in the phosphorylated state, exogenous glucosamine, which readily enters the cell by the glucose carrier [116] must be phosphorylated by glucokinase (#17) or hexokinase (#7) and acetylated by an acetyltransferase (#18) for subsequent metabolism: the phosphorylation step is completely inhibited *in vitro* by 10 mM glucose or N-acetylglucosamine and these steps could be rate-limiting in the metabolism of glucosamine [118]. After action of a mutase (#19) to produce the 1-phosphate derivative, the last enzyme of the pathway is N-acetylglucosamine-1-phosphate pyrophosphorylase (#20) which has recently been purified from pig liver: its dimeric structure suggests some regulatory properties [119]. Finally, N-acetylglucosamine released by glycoprotein breakdown can be recycled [112].

Galactosamine

As compared to other sugars, labeled galactosamine was very little used for metabolic studies. It is thought to use the enzymes of the galactose pathway. However, a N-acetylgalactosamine kinase (#21), distinct from galactokinase (#1), has been recently purified from the kidney [120], which could be used for recycling. Injected galactosamine is for the major part converted to glucosamine found in glycoproteins [121] by a specific epimerase (#22).

The main laboratory use of galactosamine is the production of hepatitis: galactosamine is specifically taken up by the liver and, by depletion of the UTP pool, induces lesions mimicking those of viral hepatitis [122], at dosages around 400 mg/kg body weight. The mechanism relies on alteration of RNA synthesis, of glucose metabolism [123] but also of alterations in protein glycosylation [124,125], especially at the level of adhesion molecules and neutrophil homing in the liver [126]. Various compounds or nutrients such as glutamine [127] can protect from galactosamine-induced hepatitis by unknown mechanisms.

Sialic acids

Neuraminic acid metabolism displays some characteristics, recognized for a long time, which distinguish it from the metabolism of other sugars [128, 129]: it is bound as cytidine monophosphate (and not as a diphosphate) from the free sugar (not from the 1-phosphate form) and the condensation by a specific synthase (#23) takes place in the nucleus [130,131], not in the cytosol. Neuraminic acid is produced from glucosamine by UDP-GlcNAc 2-epimerase (#24), which liberates the free N-acetylmannosamine. The enzyme is inhibited by CMP-NeuAc [132]. It has been purified from the kidney where it appears to be a renin binding protein [133]: physiological or regulatory significance of this property is not yet known. N-acetylmannosamine can also be directly produced from N-acetylglucosamine. The phosphorylation to N-acetylmannosamine-6-phosphate by a specific kinase (#25) could be a regulatory step, but the product can also be obtained by epimerisation of N-acetylglucosamine-6-phosphate [128]. The addition of pyruvate from glycolysis-derived phosphoenolpyruvate by a specific enzyme (#26) converts N-acetylmannosamine-6-phosphate to N-acetylneuraminic acid 9-phosphate which is converted to free N-acetylneuraminic acid by a specific phosphatase (#27). N-acetylmannosamine is frequently used as a sialic acid precursor: the addition of this sugar to cell cultures enhances the amount of CMP-NeuAc [134]. After glucosamine injection 25–30% of protein-bound radioactivity is found in the form of sialic acid, while the remainder essentially consists of glucosamine [113, 115]. Postsynthetic modifications of N-acetylneuraminic acid include O-acetylation or transformation to N-glycolyl form by a recently well characterized hydroxylase [135, 136].

In vivo, in 20-day-old fasted mice, 98% of injected N-glycolyneuraminic acid is found in urine within 6 h after oral ingestion, and 90% in the urine within 10 min after iv injection [137]. In the same conditions, N-acetylneuraminic acid exhibits similar behavior [138]. The authors conclude that dietary sialic acids cannot be used for glycosylation and there is no evidence for a significant recycling of the sugar. However, in suckling animals, sialic acid from milk could be nutritionally important: from dietary sialyllactose or NeuAc, 30% of the labeled sugar is retained in the tissues [139]; in rat pups, exogenous NeuAc could be responsible for 7–19% of neuraminic acid found in brain gangliosides [140]. The coincidence of high amounts of sialic acid containing oligosaccharides in colostrum and early milk with the high need for sialic acid during early brain development is considered as indicative of the usefulness of milk sialooligosaccharides for glycosylation. Indeed, supplementation of bovine milk with Neu5Gc has been suggested for feeding newborn calves [141] and human newborns [142]. Similarly, in human milk, the decrease in the sugar content during the course of lactation is more drastic for sialic acid (–71%) than for N-acetylglucosamine (–56%) or fucose (–35%)[143]. Though the main biological functions of milk oligosaccharides lie in anti-infective properties and in a role as dietary fibers for the newborn [144, 145], usefulness as glycosylation substrate has been suggested, as a programmed adaptation to the infant dietary requirements [143].

Overview, questions and hypotheses

From this survey, it appears that most of the old studies devoted to glycoprotein sugar metabolism were designed to unravel metabolic pathways and characterize enzymatic activities, not for nutritional purposes. However, some interesting general conclusions can be drawn concerning metabolic fate and usefulness of exogenous preformed sugars.

1. All glycoprotein sugars can derive from glucose; in some instances (sialic acid), many accessory routes can be used to produce the specific sugars. However, enzymes do exist for the direct activation of specific sugars which could be metabolically more economic than complete synthesis from glucose and allows sugar recycling.

2. The regulatory effects of intermediate or final compounds on the enzymes of the endogenous pathways have been characterized in cell-free systems and some enzymes have been suggested to be rate-limiting *in vivo*. From inhibition studies *in vitro*, it could be suggested that exogenous supply of specific sugars decreases their endogenous production. However, the relevance of such studies to physiological conditions in the whole organism largely remains to be confirmed.

3. Extracellular preformed sugars readily enter the cell *via* non-specific or specific carriers and can be directed toward glycoprotein synthesis.

4. Therefore, in the whole animal, despite oxidation or urinary elimination, a non-negligible part of the injected or (more rarely) ingested specific sugar can be incorporated into glycoproteins.

5. Though many of the enzymes display reversible action *in vitro*, there are limited interconversions into other sugars *in vivo*, with the exception of galactosamine. In some way, there seems to exist some kind of channeling either to oxidation or to direct incorporation without changes into glycoproteins.

6. Dietary free sugars are readily used by the body, but dietary protein-bound sugars can be also used for glycosylation; for obvious technical reasons in the difficulty of obtaining sufficient labeled substrates, this last point is much less documented [82, 108, 117]. It suggests either the existence of intestinal enzymatic activities able to cleave glycans, or the direct absorption of glycans further degraded inside the cells, or the intervention of bacterial microflora, some of the released sugars escaping fermentation and being absorbed in the colon.

7. Metabolic use for glycosylation would be only a part of the role of exogenous complex glycans or oligosaccharides. A major part could lie in the intestine and colon

physiology as dietary fibers and protection against bacteria, which may lead to pharmacological developments [146].

8. The concomitant supply of a large dose of the unlabeled sugar together with the labeled tracer drive the major part of the tracer to urinary elimination or oxidation. Overload is eliminated either in native form or, at least in the case of galactose and lactose, as complex oligosaccharides obtained by action of glycosyltransferases on the sugar [147]. This fact indicates that, if there is an interest in exogenous supply, the concerned amounts are low. Content of specific sugars in foods is poorly documented, due to their low amounts relative to major sugars such as glucose, galactose, lactose, sucrose or fructose. GlcNAc and GalNAc have been found in commercial milks (35–70 mg/L) [148]; sialic acid, GlcNAc and fucose have been characterized in human milks [143].

9. *In vivo* recycling of specific sugars provided by glycoprotein catabolism is suggested for all sugars with the exception of sialic acids. Specific enzymes for this purpose have been characterized in some instances.

10. With the exception of galactose, serum concentrations of specific sugars increased in diabetes mellitus, suggesting higher fluxes in hyperglycemia and a normal control of these fluxes by glucostatic hormones such as insulin. Since these increases have been implicated in the mechanisms of insulino-resistance and diabetic complications, supplementation with these sugars could have deleterious effects. However, differences in metabolic fate according to the administration route do not preclude interest in the enteral administration or in some pathological conditions apart from diabetes.

11. Specific sugars, by means of protein glycosylation or by other means such as competition or interaction with carriers, lectins, receptors [149], could exhibit actions on other metabolisms. In this way, it is not surprising that excessive exogenous sugars can exert adverse or toxic actions. Amounts currently used in such experiments are not relevant to current nutrition, but constitute indicative limits of what must not be done in nutrition.

12. Results from animal studies cannot be applied to man without caution. Differences in oxidation capacities (fucose) or in regulations of the pathways have been demonstrated in some cases. For instance, in the rabbit, contrary to man, galactose infusion induces a rise in serum insulin level [152].

Though there is still much room for progress in the molecular characterization of the pathways of sugars involved in glycosylation, the knowledge accumulated so far at the molecular level or *in vivo* in the animal is clearly out of proportion as compared to what is known in man *in vivo*. In view of the possible impact of such a knowledge for artificial nutrition in pathological conditions, and perhaps for the future development of 'nutraceutical' products, extension of research in this field appears to be highly desirable. Since the long-term health effects of most of the more important nutrients are just beginning to be understood, it seems more advisable to gain insights into the metabolic fate of these specific sugars.

Previously [1], we proposed two general hypotheses as a rationale for the study of the nutritional regulation of glycosylation, which are still valid: first, due to the complexity of the glycosylation pathway, it utilizes a large part of energy and nutrients; second, some nutrients could exert regulatory roles at various levels of the pathway. So, it would be interesting: i) to quantify endogenous fluxes of specific sugars and their regulation in various physiological and nutritional conditions; ii) to verify, in nutritionally relevant conditions, whether exogenous preformed sugars could be preferential substrates for glycosylation or could exhibit some regulatory actions on the pathway; and iii) to test whether such a supply induces subtle modifications in glycoprotein microheterogeneity or biological properties.

Increasing development of substrates labeled with stable isotopes together with highly sensitive analytical methods now offers the opportunity to perform such studies in the normal or ill man, in ethically acceptable conditions. The studies reported in the last part of this paper were undertaken as a prerequisite for the development of human investigations.

Use of stable isotopes in research on glycosylation

Since methodologies using stable isotopes had never been applied to the study of glycosylation in man, preliminary experiments for technical validation were carried out in the rat by study of the incorporation of stable isotopes into serum glycoproteins and, due to the importance of glycosylation in intestine [1], into intestinal glycoproteins. It is noteworthy that some of the dietary factors which are trophic for the intestinal mucosa, interfere with some steps of the glycosylation reactions: glutamine [151], nucleotides [152], EGF [153, 154], for the action of which glutamine is essential [155].

Glycoprotein sugars were isolated from serum or intestinal mucosa and derivatized to alditol acetates; it has been verified that the derivatization step did not induce isotopic discrimination [156]. Alditol acetates were analyzed by gas chromatography-isotope ratio mass spectrometry. Sample preparation and analysis is therefore more tedious than simple radioactivity counting. However, this is compensated for by clear identification of labeled sugars in a single run together with the determination of sugar amount and isotopic abundance. The precision of the method (< 3% for neutral sugars) allows to discriminate an isotopic enrichment as low as 0.001% carbon 13 above baseline value [157]. With a weekly continuous feeding of rat with naturally ^{13}C-enriched corn starch, a regular and significant enrichment in glycoprotein neutral sugars could be detected, of the same order of magnitude for galactose and fucose while the enrichment of mannose was lower and delayed [157]. However isotopic enrichment using natural labeling remains low: calculations suggest that a too large amount

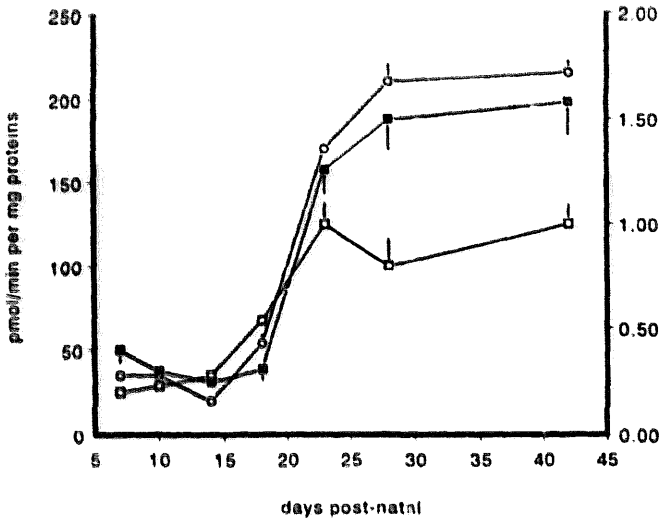

Fig 2. Evolution of the ^{13}C isotopic enrichment of serum glycoprotein sugars after ingestion of stable isotope labeled glucose. APE (atom percent excess): excess of ^{13}C above the baseline value, calculated as the difference between the ratio $^{13}C/(^{12}C+^{13}C)$ at each time and the ratio $^{13}C/(^{12}C+^{13}C)$ at time 0. Circles, glycoprotein galactose; squares, glycoprotein mannose. Labeled glucose was ingested together with 50 g of unlabeled glucose; full symbols, ingestion of 300 mg labeled glucose; open symbols: ingestion of 700 mg labeled glucose.

of corn starch would be required in human experiments. Thus, we tested the fate of artificially highly enriched glucose (0.2% in the diet) fed to the rat during a 5-h period [158]. As compared to the previous experiment, isotopic enrichment in glycoprotein sugars was 2 to 3 times higher, peaked early (4 h after the introduction of the labeled diet), and remained at a plateau up to 32 h despite a return to a non-labeled diet at time 5 h, suggesting recycling of labeled sugars. In the two types of experiments, the transitory addition to the diet of a low dose of a specific sugar such as mannose or galactose (20 to 200 mg for 250-g rats) significantly modified glycoprotein sugar enrichment, suggesting a stimulatory action on glycan synthesis. Thus, these studies in animals demonstrated that the methodology might be applied to glycoprotein sugar metabolism and also indicated possible regulatory roles of specific sugars in this metabolism.

Consequently, preliminary studies were performed in two healthy volunteers. After an overnight fast, subjects ingested either 300 or 700 mg of ^{13}C-enriched glucose (> 99% ^{13}C) together with 50 g of corn glucose diluted in 200 mL water, in order to suppress endogenous glucose production and to 'normalize' endogenous synthesis of glycoprotein sugars. Blood samples were drawn at regular intervals. ^{13}C enrichment of serum glycoprotein galactose and mannose are shown in figure 2. Due to a low fucose content, the isotopic abundance of fucose could not be accurately determined in these experiments. Isotopic enrichment was at the same level for galactose and mannose, but the enrichment for mannose displays a 3-h shift: this delay includes the metabolic conversion of glucose but also could partly represent an *in vivo* estimation of the upper limit for the duration of hepatic glycan maturation from mannose incorporation into glycan core to fixation of ante-terminal galactose. A peak of radioactivity in serum glycoproteins was observed 3 h after intraperitoneal injection of radioactive fucose in the rat [84]. Moreover, subjects ingested at 12:00 a lunch meal consisting of pastas (unlabeled wheat starch): such a massive arrival of unlabeled glucose did not induce a sharp decline in isotopic enrichment.

Conclusions

The metabolism of specific sugars used for protein glycosylation appears to be complex: biochemical pathways have been delineated and most of the enzymes involved have been characterized. However, regulations in these pathways are not yet satisfactorily known, especially *in vivo* in man. The use of stable isotope methodology could be interesting to gain insights into these regulations. Beside fundamental knowledge on a major biological event, such studies could have practical consequences in artificial nutrition (enteral or parenteral) for which glucose (or its polymers) represents the only source of glucides: sufficient for energy requirements of the organism, this might be not sufficient in a qualitative way for glycosylation. In the state-of-the-art, currently used parenteral nutrition frequently induces liver dysfunction [159]; it has been shown that in some situations, more complex saccharide mixtures containing glucose, fructose and xylitol could improve clinical situations as compared to glucose alone [160]. In this way, specific sugars could represent interesting non-essential nutrients.

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